EXHIBIT F

Assessment of the efficacy of a single dose of a recombinant vaccine against West Nile virus in response to natural challenge with West Nile virus-infected mosquitoes in horses

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Objective—To determine the onset of immunity after IM administration of a single dose of a recombinant canarypox virus vaccine against West Nile virus (WNV) in horses in a blind challenge trial.

Animals—20 mixed-breed horses.

Procedure—Horses with no prior exposure to WNV were randomly assigned to 1 of 2 groups (10 horses/group). In 1 group, a recombinant canarypox virus vaccine against WNV was administered to each horse once (day 0). The other 10 control horses were untreated. On day 26, 9 treated and 10 control horses were challenged via the bites of mosquitoes (*Aedes albopictus*) infected with WNV. Clinical responses and WNV isolation were monitored for 14 days after challenge exposure; antibody responses against WNV after administration of the vaccine and challenge were also assessed in both groups.

Results—Following challenge via WNV-infected mosquitoes, 1 of 9 treated horses developed viremia. In contrast, 8 of 10 control horses developed viremia after challenge exposure to WNV-infected mosquitoes. All horses seroconverted after WNV challenge; compared with control horses, antibody responses in the horses that received the vaccine were detected earlier.

Conclusions and Clinical Relevance—In horses, a single dose of the recombinant canarypox virus-WNV vaccine appears to provide early protection against development of viremia after challenge with WNV-infected mosquitoes, even in the absence of measurable antibody titers in some horses. This vaccine may provide veterinarians with an important tool in controlling WNV infection during a natural outbreak or under conditions in which a rapid onset of protection is required. (*Am J Vet Res* 2004;65:1459–1462)

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West Nile virus (WNV) is an arthropod-borne, enveloped, positive-stranded RNA virus that is transmitted by mosquitoes. Birds are the principal vertebrate hosts, but the virus can occasionally cause disease in horses and humans. West Nile virus first appeared in the United States in 1999 in New York, where it was associated with a small number of illnesses in horses and humans.[1-4] Since then, the virus has spread across most of the United States and into Canada and Mexico, and WNV is now considered to be endemic in North America. In 2002, WNV infection and associated clinical disease was diagnosed in more than 14,000 horses in the United States, of which approximately 33% died.[5-7] In 2003, there were fewer reports of infected horses (between 4,000 and 5,000), although mortality rates seemed to remain constant.[8,9] As the virus has spread westward in the United States, there has been an apparent decrease in the incidence of WNV-associated disease among horses in previously affected areas. An important exception to this is the mid-Atlantic states, where the incidence of WNV-associated disease in horses in 2003 was 5 times as great as that reported in 2002.[5,6] Thus, the virus and disease remain unpredictable in terms of epidemiology, virulence, and response to environmental factors.

At present, there is no effective treatment protocol for WNV infection in horses and control of the spread of infection relies on vaccination and mosquito management. Although the use of vaccines experimentally in several species has been described,[10] there are only 2 commercially available vaccines: 1 prepared with an inactivated whole virus and another that incorporates recombinant canarypox virus expressing prM/E proteins of WNV. In another study[11] by our group, 2 doses of the recombinant canarypox virus-WNV vaccine provided protection against WNV infection that lasted for at least 12 months in WNV-naive horses. The purpose of the study of this report was to determine the onset of immunity after IM administration of a single dose of a recombinant vaccine against WNV in horses in a blind challenge trial.

Materials and Methods

Animals—Twenty mixed-breed horses (age, 2 to 8 years) of either sex from a commercial herd were included in this study. None of the horses had detectable antibody against WNV or St Louis encephalitis virus via plaque reduction neutralization testing at the commencement of the trial. Horses were individually housed in environmentally con-

trolled stalls at a research facility in Missouri until day 13 after vaccination, at which time they were transported to Colorado State University. At this location, horses were housed in a biosecurity level-3 containment building (2 horses/room) until the end of the trial. At both sites, horses were fed a diet of pelleted concentrates and maintained in accordance with animal use and care guidelines of the Merial and Colorado State University Institutional Animal Care and Use Committees.

Vaccine against WNV—A commercial preparation of freeze-dried vaccine[4] that contains recombinant canarypox virus expressing the prM/E genes derived from a 1999 New York isolate of WNV was used. The vaccine was diluted with cross-linked acrylic acid polyalkenyl ether divinyl glycol[5] adjuvant to provide 10^6 tissue culture infectious dose 50% ($TCID_{50}$) of vaccine virus/dose.

Experimental design—Horses were randomly assigned to 1 of 2 groups (10 horses/group). On day 0, horses in group 1 received a single dose (1 mL) of recombinant canarypox virus-WNV vaccine. The vaccine was not administered to horses in group 2 (control horses). For personnel safety reasons, 1 horse in group 1 was excluded from the study just prior to challenge exposure.

On day 26, all horses (9 treated and 10 control horses) in the study were challenged with mosquitoes infected with WNV, as previously described.[12] Briefly, each horse was challenged by the bites of 3 to 11 (mean, 7) mosquitoes (*Aedes albopictus*) that had been previously inoculated with approximately 500 to 1,000 plaque-forming units (pfu) of the NY99-4132 WNV isolate. Mosquitoes were allowed to feed on horses for a period of 10 to 20 minutes. After the feeding period, 3 engorged mosquitoes from each horse were collected, homogenized, and evaluated for virus by plaque assay. The results of the back-titration indicated that the engorged mosquitoes contained 2.9×10^7 to 1.2×10^9 pfu of WNV/mosquito.

Blood samples were collected from all horses on days 0, 7, 14, 21, 26, 33, and 40 for assessment of serum antibodies against WNV. On days 0 and 26, samples were obtained prior to the administration of vaccine and mosquito challenge, respectively. Sera were prepared from the blood samples and stored at −80°C until analyzed to determine antibody responses. In addition, blood samples were obtained twice daily from all horses for 13 days following challenge (days 26 through 39) and once on day 40 to provide sera for virus isolation. On day 26, the first set of samples was obtained prior to challenge and the second set was obtained after challenge. Serum samples were stored frozen at −80°C until virus isolation was performed.

Physical examinations were performed on each horse twice daily on days 26 through 39 and once on days 24, 25, and 40. Examinations included measurement of rectal temperature and observations of signs of depression or anxiety, ataxia, head shaking, muscle fasciculation, reluctance to move, and lip twitching (each recorded as present or absent). At the end of the trial, horses were euthanatized by IV administration of an overdose of sodium pentobarbital and the carcasses were incinerated.

The persons performing the laboratory analysis and clinical observations were unaware of the treatment assignments. Personnel involved in the vaccination procedure were not involved in the evaluation of clinical signs and laboratory analyses.

Virus isolation via plaque assay—Serum samples and mosquito homogenates were assessed for live virus via titration in a plaque assay, as previously described.[12] Briefly, 0.1 mL of sample was added to a monolayer of Vero cells in a 6-well culture plate and incubated for 1 hour at 37°C in an atmosphere containing 5% carbon dioxide. Cells were overlaid with 2 mL of 0.5% agarose in Dulbecco's modified Eagle medium (without phenol red) supplemented with 5% fetal bovine serum and antimicrobials. After 48 hours of additional incubation, a second 2-mL overlay of the same solution with 0.004% neutral red was added to the cells. Plaques were counted on days 3, 4, and 5 of incubation and expressed as number of pfu per milliliter of serum.

Determination of antibody responses—Antibody response against WNV was evaluated by use of a plaque reduction neutralization test. Titration of sera was done in blinded fashion at Colorado State University, as described previously.[12] Briefly, sera were heat-inactivated at 56°C for 30 minutes, and serial 2-fold dilutions of those sera were mixed with an equal volume (0.1 mL) of a preparation containing 200 to 300 pfu of the NY99-4132 strain of WNV. After incubation at 4°C for approximately 16 to 20 hours, 0.1 mL of each mixture was laid on Vero cell monolayers in 6-well plates and processed as for the plaque assay. Neutralization end points were recorded as the highest dilution of serum with which there was a 50% reduction of plaques (ie, detection of 100 to 150 pfu). A titer ≥ 1:5 was considered to represent a positive antibody response against WNV.

Statistical analyses—Comparison of the incidence of WNV viremia between the groups was analyzed by use of a Fisher exact test. A repeated-measures ANOVA with factors of group, day, and group-day interaction was constructed to analyze the net change in body temperature from baseline; the baseline temperature was defined as the mean value of rectal temperatures recorded on prechallenge days 24, 25, and 26. All statistical analyses were conducted by use of computer software.[5] Significance was based on 2-tailed tests of the null hypothesis resulting in P values ≤ 0.05.

Results

Antibody responses—On days 0 and 7, none of the 9 horses that received vaccine had detectable antibodies against WNV. On days 14 and 21, 6 and 7 of 9 horses in group 1 had detectable antibodies against WNV, respectively. On day 26 (challenge day), 6 of 9 horses in group 1 had detectable antibodies against WNV. The antibody titers determined via plaque reduction neutralization testing ranged from 1:5 to 1:80. None of the control horses had detectable antibodies against WNV at any time prior to the challenge exposure. Following challenge with WNV-infected mosquitoes, all horses seroconverted, but anamnestic responses in the treated horses appeared earlier than those in the control horses (Figure 1).

Assessment of viremia following WNV challenge—On days 26 to 40, blood samples were obtained from all horses to provide sera for virus isolation. On days 26 through 39, samples were collected twice daily; horses were considered to be viremic if virus was isolated from 1 or both serum samples obtained on that day. Among the control horses, viremia was detected in 2 horses 24 to 48 hours after exposure to WNV-infected mosquitoes and in 4 horses 48 to 72 hours after challenge. Eight of 10 control horses had detectable viremia from 72 to 120 hours after challenge, with peak virus titers of $10^{1.5}$ to $10^{2.2}$ pfu/mL. From 120 to 144 hours and 144 to 168 hours after challenge, viremia was detected in 7 and 2 control horses, respectively. Virus was not isolated after 168 hours postchal-

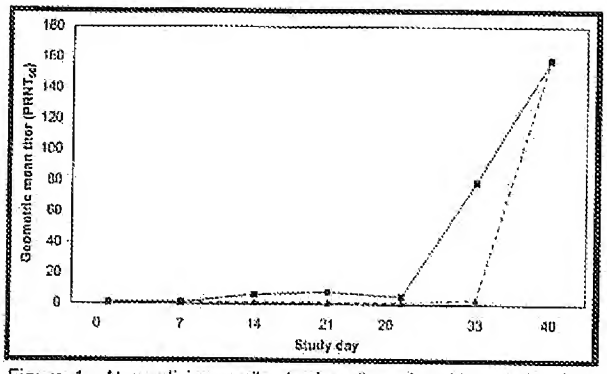

Figure 1—Neutralizing antibody titers* against West Nile virus (WNV) in 10 horses† that were administered IM a single dose of recombinant canarypox virus-WNV vaccine (squares) and 10 untreated control horses (triangles). Vaccine was administered on day 0 and on day 26; 9 treated and all control horses were challenged via the bites of WNV-infected mosquitoes. Sera were obtained on study days 0, 7, 14, 21, and 26 (before challenge) and on study days 33 and 40 (after challenge). *The neutralizing antibody titers were represented as the highest serum dilution yielding a 50% reduction in plaque numbers ($PRNT_{50}$). †For personnel safety reasons, 1 horse in the treated group was excluded from the study just prior to challenge exposure.

lenge in the control horses. Only 1 of 9 horses in group 1 developed viremia and was viremic 72 to 96 hours and 144 to 168 hours after challenge, with a peak virus titer of $10^{1.6}$ pfu/mL. This horse had mounted a neutralizing antibody response after vaccination (peak antibody titer of 1:20), which was still present at the time of challenge (titer of 1:5). The incidence of viremia in the group 1 horses was significantly ($P = 0.006$) lower than in the control horses.

Clinical signs of disease in horses after WNV challenge—During physical examinations performed on days 24 through 40, clinical signs of disease were not observed in any of the horses, with the exception of 1 control horse that transiently had a high temperature (38.8°C) 3 days after challenge. There was no significant ($P = 0.96$) difference between the groups with respect to temperature after challenge.

Discussion

The recent outbreaks of WNV infection in horses have raised the question of how quickly protection can be induced following vaccination. The results of the present study have indicated that a single dose of the live recombinant canarypox virus-WNV vaccine provided protection against development of WNV viremia within 26 days of IM administration in horses. To our knowledge, this is the only commercially available vaccine for which a rapid onset of protective immunity against a natural WNV challenge after 1 injection has been demonstrated.

An inactivated vaccine against WNV is commercially available and has been shown to be effective in preventing WNV viremia in needle challenge experiments in horses.[11] According to the manufacturer's recommendations,[4] this vaccine has to be administered in 2 doses at an interval of 3 to 6 weeks apart to induce immunity. Indeed, results of a recent study[14] indicated that 277 of 589 (47%) WNV-infected horses in Colorado and Nebraska had received at least 1 dose of the inactivated vaccine prior to developing clinical signs of disease.

None of the horses challenged with WNV in our study developed clinical signs of disease associated with WNV infection. This was not unexpected because findings of previous studies[11,12] in horses experimentally infected with WNV yielded similar results and, under field conditions,[15] the percentage of horses exposed to WNV that had clinical signs of disease has been estimated to be 10%. In the absence of clinical signs, viremia is considered to be the most consistent indicator of WNV infection in horses; monitoring the development of viremia by use of virus isolation is currently accepted as a means of assessing the efficacy of vaccines against WNV.[11,13,16] The magnitude and duration of viremia observed in the horses of our study were consistent with findings of previous studies.[11,12]

Although the antiviral and immunomodulating properties of poxviruses have been well documented,[17] it is highly unlikely that the protection afforded by the recombinant canarypox virus vaccine against WNV was a result of the nonspecific antiviral activity of the canarypox virus. Results of earlier studies indicate that the canarypox vector alone or canarypox virus expressing an unrelated gene did not provide protection against canine distemper virus infection in ferrets,[18] equine herpesvirus infection in horses,[19] and, in our experience, equine influenza virus infection in horses. Moreover, because it is well accepted that the immunomodulatory properties of poxviruses are mediated by short-lived cytokines, we would not expect the nonspecific immunity to provide protection against WNV infection for as long as 26 days after vaccination. Therefore, we believe that the effective viral clearance and the rapid onset of immunity against WNV provided by a single administration of the live recombinant canarypox virus-WNV vaccine in the group 1 horses reflects strong specific priming of their immune systems.[20] The protective immunologic responses to WNV infection in horses have not been elucidated, but cell-mediated immunity may be fundamental to effective viral clearance. In a study[21] of B-cell–deficient and T- and B-cell–deficient mice, administration of polyclonal antibody against WNV was ineffective in eliminating virus from WNV-infected mice, which emphasizes the key role of cell-mediated immunity in viral clearance. In the present study, 3 of 9 horses that received 1 dose of the vaccine had no antibodies detected via plaque reduction neutralization testing at the time of challenge with WNV-infected mosquitoes, yet they did not develop WNV viremia, which is indicative of cell-mediated viral clearance.

The vaccine vector used in the study of this report shares several features with other poxviruses, including a capacity to accept large segments of foreign DNA and thermo- and genetic stability; these characteristics make poxviruses ideal vectors for delivering foreign antigens from a wide variety of pathogens to the host immune system.[7] Canarypox virus recombinants authentically express the inserted foreign genes in the absence of productive viral replication,[22] which provides a built-in safety feature for vaccination purposes because there is no potential for transmission of the vaccine vector.

It is expected that WNV infection will continue to be a concern for horse owners and veterinarians in the United States, with further outbreaks of disease in endemic areas and detection of new foci of infection in previously unaffected regions. The results of the study reported here indicate that the recombinant canarypox virus-WNV vaccine induced a rapid onset of immunity against WNV after a single administration in horses, which can be of crucial importance when exposure to WNV may closely follow vaccination. This vaccine may provide veterinarians with an important tool in controlling WNV infection during a natural outbreak or under conditions in which a rapid onset of protection against WNV infection and associated disease is required.

[a]Recombitek equine West Nile virus vaccine, Merial Ltd, Duluth, Ga.
[b]Carbopol, BF Goodrich Co, New York, NY.
[c]SAS-PC, version 8.2, SAS Institute Inc, Cary, NC.
[d]Fort Dodge Animal Health, Fort Dodge, Iowa.

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